Supplementary Information

Novel oxysterols observed in tissues and fluids of AY9944-treated rats – a model for Smith-Lemli-Opitz Syndrome

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Contents Page

Table S1. ^{1}H-NMR chemical shifts of new oxysterols isolated from brain tissues of AY9944-treated rats.[a]

Proton	**4α-OH-7-DHC**	**4β-OH-7-DHC**	**24-OH-7-DHC**
3	3.39 (m)	3.66 (m)	3.64 (m)
4	4.05 (br t, 7.7)	4.27 (br s)	2.47 (ddd, 14.4, 4.7, 2.2) 2.29 (br t, 12.9)
6	6.02 (dd, 5.8, 2.2)	5.92 (d, 5.5)	5.57 (dd, 5.7, 2.4)
7	5.52 (dt, 5.8, 2.5)	5.49 (dt, 5.5, 2.7)	5.39 (dt, 5.7, 2.7)
9	2.02 (t, 9.1)	1.96 (m)	1.97 (m)
18	0.62 (s)	0.60 (s)	0.62 (s)
19	0.98 (s)	1.11 (s)	0.94 (s)
21	0.94 (d, 6.5)	0.94 (d, 6.5)	0.97 (d, 6.6)
26	0.86 (d, 2.7)	0.86 (d, 2.6)	0.90 (d, 6.8)
27	0.88 (d, 2.7)	0.87 (d, 2.6)	0.93 (d, 6.8)

[a] All in $CDCl_3$ at 600 MHz

Table S2. ^{13}C-NMR chemical shifts of new oxysterols isolated from brain tissues of AY9944-treated rats.[a]

Carbon	**4α-OH-7-DHC**	**4β-OH-7-DHC**	**24-OH-7-DHC**[b]
3	75.8	71.9	70.7
4	75.3	74.7	41.1
5	141.5	141.0	140.1
6	116.9	126.0	119.8
7	116.1	116.4	116.5
8	142.4	145.6	N.A.[c]
9	46.8	46.9	46.6
10	38.6	36.1	N.A.[c]
13	43.1	43.3	43.1
14	54.7	54.8	54.8
17	56.0	56.0	55.8
18	12.0	12.0	12.1
19	17.8	18.2	19.3
20	36.3	36.3	36.6
21	19.0	19.0	19.3
22	36.3	36.3	32.2
24	39.7	39.7	77.6
25	28.2	28.2	33.5
26	22.7	22.7	16.7
27	23.0	23.0	16.9

[a] All in $CDCl_3$ at 600 MHz; [b] Indirect values from HSQC and HMBC spectra;

[c] N.A., not assigned.

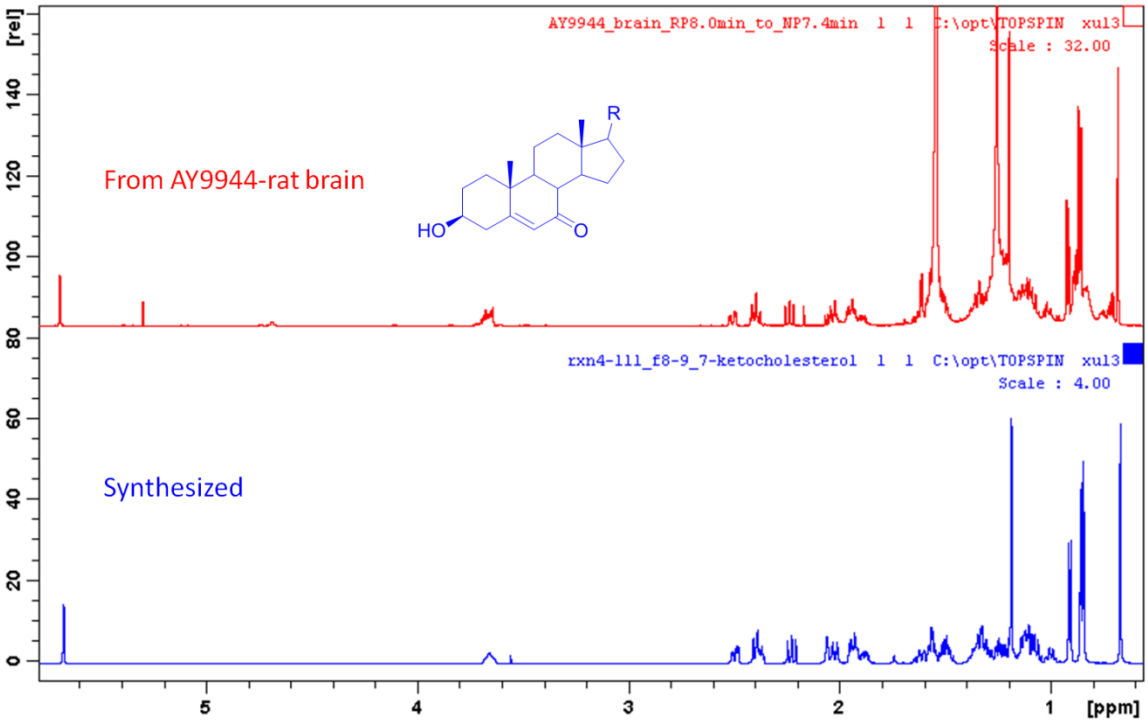


Figure S1. Comparison of ^{1}H NMR spectrum of 7kChol isolated from AY9944-rats to the synthetic standard.

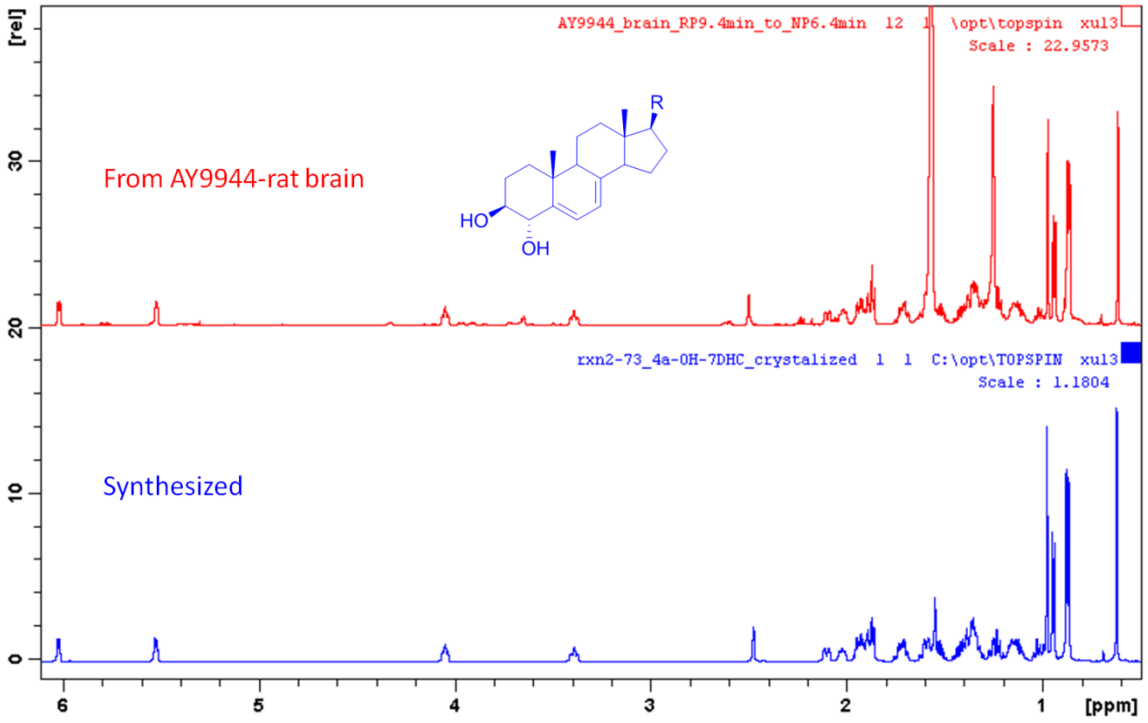


Figure S2. Comparison of ^{1}H NMR spectrum of 4α-hydroxy-7-DHC isolated from AY9944-rats to the synthetic standard.

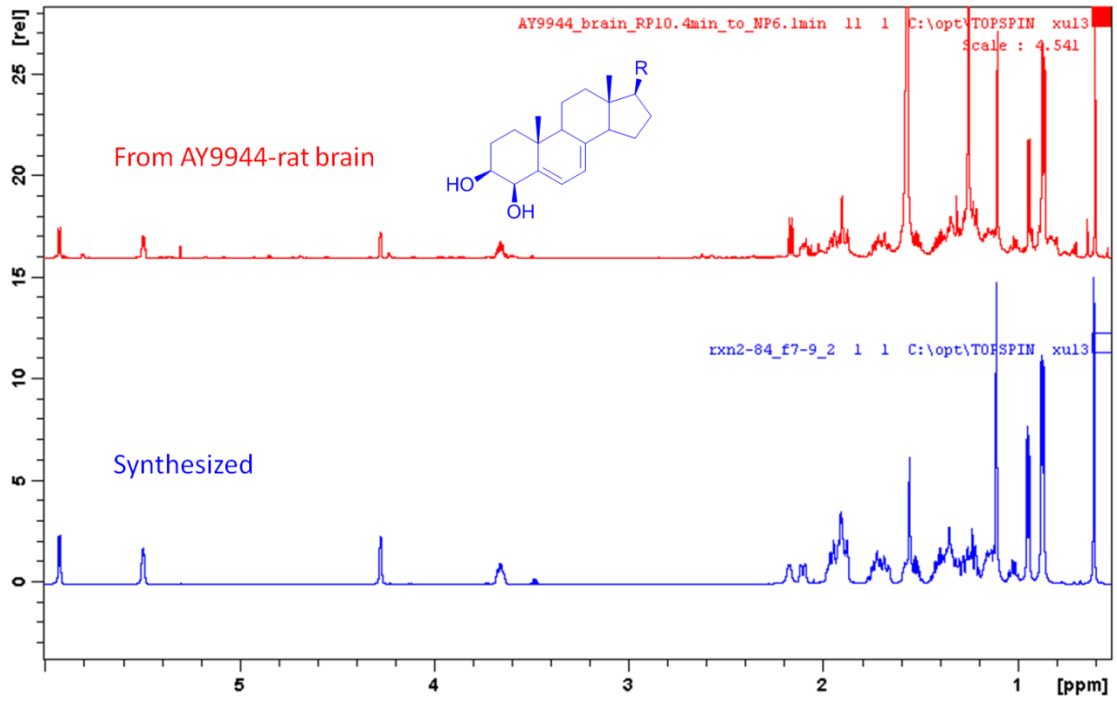


Figure S3. Comparison of ^{1}H NMR spectrum of 4β-hydroxy-7-DHC isolated from AY9944-rats to the synthetic standard.

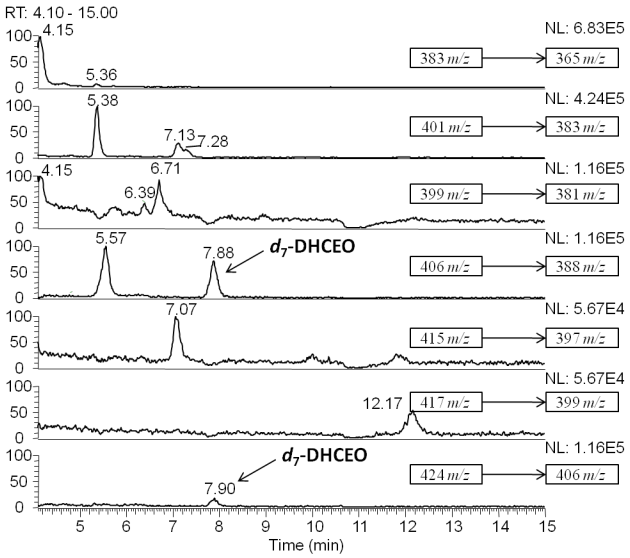


Figure S4. NP-HPLC-APCI-MS-MS chromatograms of oxysterols from liver tissue of 2-month old control rats.

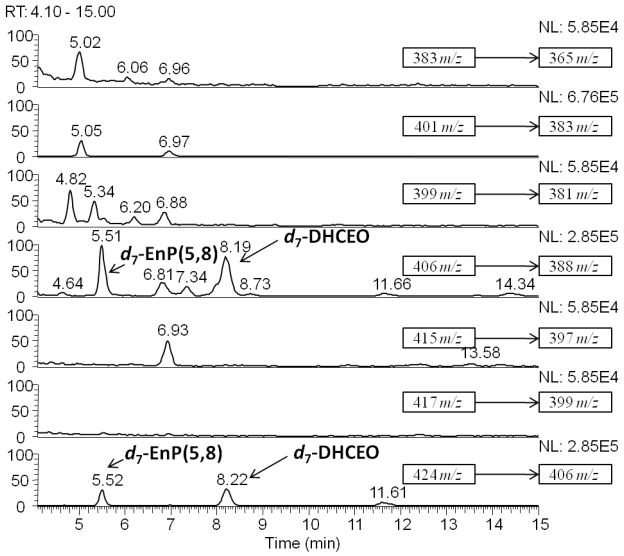
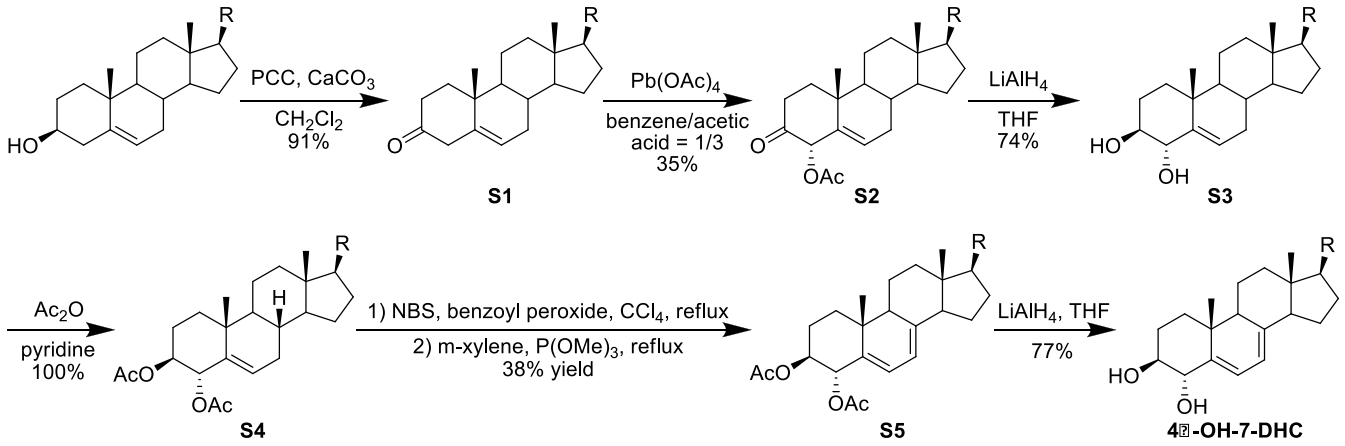
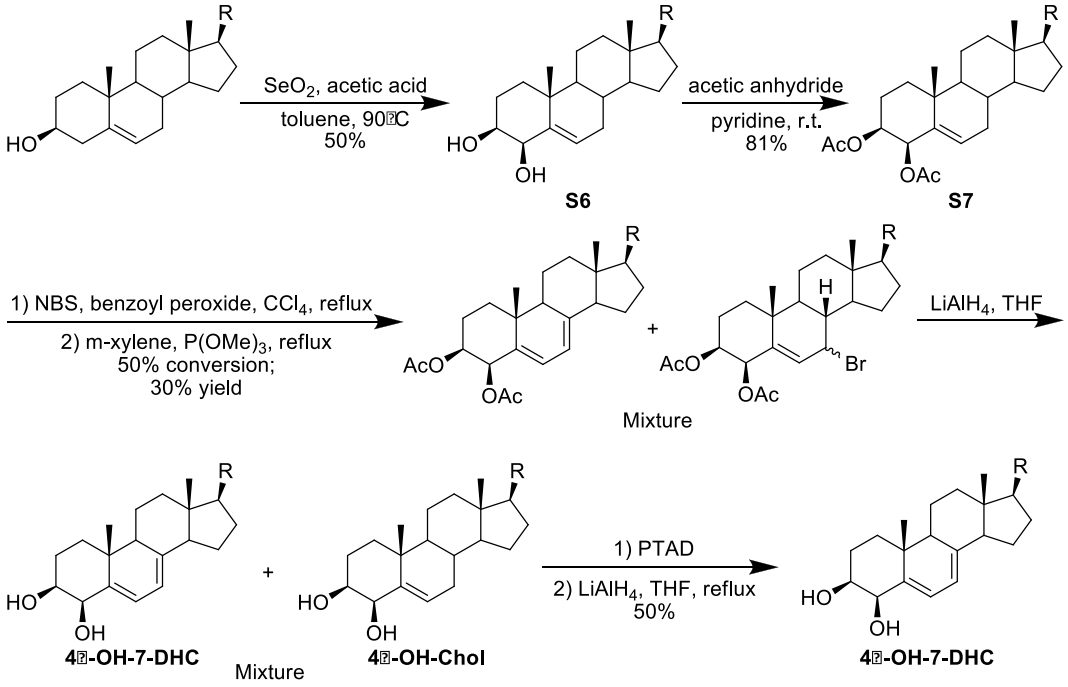


Figure S5. NP-HPLC-APCI-MS-MS chromatograms of oxysterols from KOH-hydrolyzed serum from 2-month old control rats.

Synthesis:



Scheme S1. Synthesis of 4α-hydroxy-7-DHC.



Scheme S2. Synthesis of 4β-hydroxy-7-DHC.

4α-Hydroxy-7-DHC and 4β- hydroxy-7-DHC were synthesized by modified procedures from literatures as illustrated in **Scheme S1** and **S2** (1-4). The detailed procedures were summarized below.

Cholest-5-en-3-one (S1). A solution of cholesterol (3.0 g, 7.76 mmol) in CH_2Cl_2 (40 mL) was added to a stirred suspension of pyridinium chlorochromate (5.0 g, 23.2 mmol) and $CaCO_3$ (5.0 g, 50.0 mmol) in CH_2Cl_2 (40 mL). The resulting mixture was stirred under argon for 30 min and cold anhydrous ether (400 mL) was added. The mixture was filtered through a plug of silica gel 5 times and the filtrate was evaporated to dryness to give titled product as a white amorphous solid (2.7 g, 91%, < 10% cholest-4-en-3-one), which was used in next step without further purification. NMR spectra are consistent with those of a commercial standard from Sigma-Aldrich Co.

4α-Acetoxycholest-5-en-3-one (S2). To a stirred solution of S1 (3.22 g, 8.37 mmol) in benzene (13 mL) at 15 °C was added acetic acid (33 mL) and $Pb(OAc)_4$ (4.1 g, 9.21 mmol). The reaction mixture was stirred at 15 – 20 °C for 2 hr until nearly all solid was dissolved. The reaction was then warmed up to room temperature and was left overnight. To work up the reaction, water (40 mL) was added and the resulting mixture was extracted with ether (40 mL ×2). The extract was washed with saturated $NaHCO_3$ in water, dried over $MgSO_4$, filtered and evaporated. Thus obtained solid was recrystalized in 95% ethanol to give the product as a white solid (1.3 g, 35%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.71 (s, 3H), 0.86 (d, 3H, J = 2.1 Hz), 0.87 (d, 3H, J = 2.1 Hz), 0.92 (d, 3H, J = 6.5 Hz), 1.29 (s, 3H), 2.23 (s, 3H), 2.41 (dt, 1H, J = 14.5, 3.9 Hz), 2.63 (td, 1H, J = 14.1, 5.7 Hz), 5.71 (dt, 1H, J = 5.5, 2.3 Hz), 5.89 (d, 1H, J = 2.9 Hz).^{13}C NMR (150 MHz, $CDCl_3$): δ 12.0, 18.8, 20.2, 20.9, 21.3, 22.7, 23.0, 24.0, 24.4, 28.2, 28.4, 31.6, 31.7, 35.9, 36.2, 36.3, 37.9, 38.3, 39.6, 39.7, 42.4, 49.8, 56.2, 56.6, 76.7, 119.9, 137.5, 170.0, 203.5.

4α-Hydroxycholesterol (S3). To a stirred solution of **S2** (703 mg, 1.6 mmol) in THF was added $LiAlH_4$ (120 mg, 3.2 mmol) in small portions. The resulting mixture was stirred at room temperature for 30 min. The reaction was quenched by adding water dropwise, acidified with HCl (5% in water), extracted with CH_2Cl_2, dried over $MgSO_4$, and evaporated to dryness. The crude product was recrystalized from acetone to give the product as a white solid (475 mg, 74%). The mother liquor still contains compound **S3** as the major component (by TLC), but was not further purified. ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.68 (s, 3H), 0.86 (d, 3H, J = 2.2 Hz), 0.87 (d, 3H, J = 2.2 Hz), 0.91 (d, 3H, J = 6.6 Hz), 1.02 (s, 3H), 1.79-1.91 (m, 3H), 2.02 (dt, 1H, J = 12.7, 3.6 Hz), 2.05-2.13 (m, 1H), 3.24 (td, 1H, J = 10.5, 4.1 Hz), 4.05 (m, 1H), 5.74 (dt, 1H, J = 5.4, 2.1 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 12.0, 18.9, 20.4, 21.1, 22.7, 23.0, 24.0, 24.4, 28.16, 28.20, 28.4, 31.6, 31.7, 35.9, 36.3, 36.8, 38.2, 39.7, 39.9, 42.4, 50.6, 56.3, 56.9, 75.3, 76.7, 118.0, 142.2.

4α-Hydroxycholesterol diacetate (S4). Compound **S3** (303 mg, 0.75 mmol) was stirred in pyridine (4 mL) and acetic anhydride (4 mL) at room temperature overnight. The solvent was removed under vacuum as much as possible and the residue was re-dissolved in ethyl acetate (30 mL), washed with water (30 mL × 2), saturated $NaHCO_3$ (30 mL × 3), and 5% HCl (30 mL). The organic layer was dried over $MgSO_4$, filtered through a plug of silica gel, and evaporated to dryness to give the product as a white solid (365 mg, 100%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.66 (s, 3H), 0.85 (d, 3H, J = 2.7 Hz), 0.86 (d, 3H, J = 2.7 Hz), 0.90 (d, 3H, J = 6.5 Hz), 1.08 (s, 3H), 1.63-1.73 (m, 1H), 1.78-1.88 (m, 2H), 1.92-1.98 (m, 1H), 2.01 (s, 3H), 2.09 (s, 3H), 4.70 (td, 1H, J = 10.9, 5.0 Hz), 5.45 (dt, 1H, J = 5.5, 2.1 Hz), 5.55 (dd, 1H, J = 10.2, 2.4 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 11.9, 18.8, 20.0, 21.0, 21.1, 21.3, 22.7, 22.9, 23.9, 24.4, 26.1, 28.1, 28.3, 31.4, 31.6, 35.9, 36.3, 38.2, 39.6, 39.7, 42.3, 50.4, 56.2, 56.7, 73.2, 75.5, 119.4, 137.5, 170.3, 170.5.

4α-Hydroxy-7-DHC diacetate (S5). To a refluxing solution of diacetate **S4** (585 mg, 1.2 mmol) in CCl_4 (73 mL) was added N-bromosuccinimide (267 mg, 1.5 mmol) and benzoyl peroxide (29 mg, 0.12 mmol) in one portion. The reaction mixture was boiled under reflux for 30 min, cooled to 0 °C, filtered, and evaporated to dryness. Thus obtained yellowish residue was subsequently dissolved in m-xylene (27.6 mL) and $P(OMe)_3$ (5.5 mL) was added. The resulting solution was refluxed overnight. After removing the solvent under vacuum, the crude product was purified by flash column chromatography on silica gel (elution solvent, hexanes/ethyl acetate = 10/1) to give the product as a white amorphous solid (220 mg, 38%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.60 (s, 3H), 0.86 (d, 3H, J = 2.8 Hz), 0.87 (d, 3H, J = 2.8 Hz), 0.94 (d, 3H, J = 6.4 Hz), 1.02 (s, 3H), 2.02 (s, 3H), 2.12 (s, 3H), 4.81 (td, 1H, J = 10.6, 4.6 Hz), 5.47 (dt, 1H, J = 5.8, 2.5 Hz), 5.61 (dd, 1H, J = 5.9, 2.2 Hz), 5.64 (d, 1H, J = 10.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 12.0, 17.4, 19.0, 21.0, 21.2, 21.3, 22.7, 23.0, 23.1, 24.0, 26.2,

28.16, 28.20, 36.2, 36.3, 37.0, 38.4, 39.1, 39.6, 43.1, 46.3, 54.6, 56.0, 72.7, 74.7, 115.9, 118.1, 136.1, 142.8, 170.61, 170.64.

4α-Hydroxy-7-DHC. To a solution of diacetate **S5** (220 mg, 0.45 mmol) was added $LiAlH_4$ (34 mg, 0.91 mmol) slowly. The reaction was stirred at room temperature for 20 min and was quenched by adding water dropwise, diluted with saturated NH_4Cl, extracted with CH_2Cl_2, dried over $MgSO_4$, filtered, and evaporated under vacuum. The crude product was purified by flash column chromatography on silica gel (elution solvent, hexanes/ethyl acetate = 2/1) to give pure 4α-hydroxy-7-DHC as a white amorphous solid (139 mg, 77%).^{1}H NMR (600 MHz, $CDCl_3$): δ 0.62 (s, 3H), 0.86 (d, 3H, J = 2.7 Hz), 0.88 (d, 3H, J = 2.7 Hz), 0.94 (d, 3H, J = 6.5 Hz), 0.98 (s, 3H), 1.67-1.77 (m, 2H), 1.84-1.98 (m, 5H), 2.02 (t, 1H, J = 9.1 Hz), 2.10 (ddd, 1H, J = 12.9, 4.4, 2.5 Hz), 3.39 (m, 1H), 4.05 (br t, 1H, J = 7.7 Hz), 5.52 (dt, 1H, J = 5.8, 2.5 Hz), 6.02 (dd, 1H, J = 5.8, 2.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 12.0, 17.8, 19.0, 21.1, 22.7, 23.0, 23.2, 24.0, 28.18, 28.24, 36.27, 36.29, 37.9, 38.6, 39.2, 39.7, 43.1, 46.8, 54.7, 56.0, 75.3, 75.8, 116.1, 116.9, 141.5, 142.4.

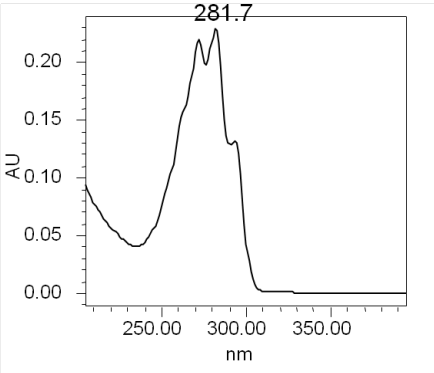
4β-Hydroxycholesterol (S6). To a solution of SeO_2 (960 mg, 8.7 mmol) in acetic acid (12 mL) under stirring at 90 °C was added a solution of cholesterol (1.2 g, 3.1 mmol) in toluene (16 mL) that was also pre-heated to 90 °C. The reaction mixture was stirred at 90 °C for another 1.5 hr. The reaction was stopped by adding NaOAc•$3H_2O$ (4.0 mg, 29 mmol). The reaction mixture separated into two layers and was stirred for additional 10 min at room temperature. The mixture was then filtered and washed with toluene (30 mL). The filtrate was washed with water (20 mL × 3), dried over $MgSO_4$, and evaporated under vacuum. Flash column chromatography on silica gel (elution solvent, hexanes/ethyl acetate = 8/1 to 4/1 to 2/1) gave product as a white solid (624 mg, 50%). ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.68 (s, 3H), 0.86 (d, 3H, J = 1.75 Hz), 0.87 (d, 3H, J = 1.75 Hz), 0.91 (d, 3H, J = 6.5 Hz), 1.77-1.97 (m, 3H), 1.97-2.15 (m, 4H), 3.56 (m, 1H), 4.13 (t, 1H, J = 1.75 Hz), 5.68 (dd, 1H, J = 4.8, 2.2 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 12.0, 18.9, 20.7, 21.2, 22.7, 23.0, 24.0, 24.4, 25.6, 28.2, 28.4, 32.0, 32.2, 35.9, 36.2, 36.3, 37.1, 39.7, 39.8, 42.5, 50.3, 56.3, 57.1, 72.7, 77.4, 129.0, 142.9.

4β-Hydroxycholesterol diacetate (S7). The reaction was carried out in a similar procedure to the synthesis of **S4** with a yield of 81%. ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.67 (s, 3H), 0.86 (d, 3H, J = 2.2 Hz), 0.87 (d, 3H, J = 2.2 Hz), 0.91 (d, 3H, 6.5 Hz), 1.13 (s, 3H), 2.01 (s, 3H), 2.06 (s, 3H), 4.74 (dt, 1H, J = 12.2, 3.9 Hz), 5.50 (d, 1H, J = 2.9 Hz), 5.81 (dd, 1H, J = 4.7, 2.0 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 12.0, 18.9, 20.6, 20.7, 21.3, 21.6, 22.67, 22.71, 23.0, 24.0, 24.4, 28.2, 28.3, 31.8, 32.2, 35.9, 36.29, 36.32, 36.9, 39.7, 39.8, 42.4, 50.4, 56.2, 56.9, 73.0, 76.1, 131.8, 138.4, 170.3, 170.5.

4β-hydroxy-7-DHC. 4β-Hydroxycholesterol diacetate **S7** was converted to 4β-hydroxy-7-DHC diacetate in a similar procedure to the synthesis of **S5** but with a yield of 30% and the presence of inseparable bromide. The mixture was reduced directly with $LiAlH_4$ to give a mixture of 4β-hydroxy-7-DHC and 4β-hydroxycholesterol. The mixture was purified following literature procedure by reacting with 4-phenyl-1,2,4-triazoline-3,5-dione (PTAD) (4). The resulting 4β-hydroxy-7-DHC-PTAD adduct was crystallized in acetone at 0 °C. Thus obtained white solid was dissolved in THF and was refluxed for 3 hr in the presence of 4 equivalent of $LiAlH_4$ with a yield of 40%. ^{1}H NMR (600 MHz, $CDCl_3$): δ 0.60 (s, 3H), 0.86 (d, 3H, J = 2.6 Hz), 0.87 (d, 3H, J = 2.6 Hz), 0.94 (d, 3H, J = 6.5 Hz), 1.11 (s, 3H), 1.63-1.79 (m, 3H), 1.84-2.0 (m, 6H), 2.10 (br d, 1H, J = 12.7 Hz), 3.66 (m, 1H), 4.27 (br s, 1H), 5.49 (dt, 1H, J = 5.5, 2.7 Hz), 5.92 (d, 1H, J = 5.5 Hz). ^{13}C NMR (150 MHz, $CDCl_3$): δ 12.0, 18.2, 19.0, 20.5, 22.7, 23.0, 23.2, 24.0, 25.4, 28.17, 28.20, 36.1, 36.3, 38.1, 39.1, 39.7, 43.3, 46.9, 54.8, 56.0, 71.9, 74.7, 116.4, 126.0, 141.0, 145.6.

UV spectra of isolated oxysterols from AY9944-treated rats (in hexanes:2-propanol = 9:1):

4α-hydroxy-7-DHC:



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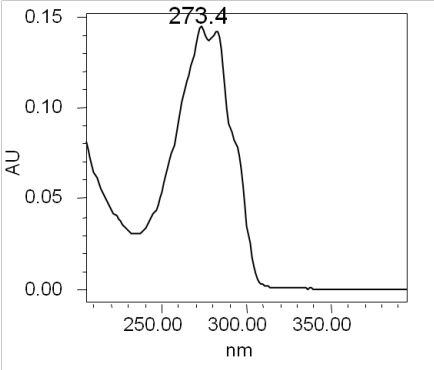

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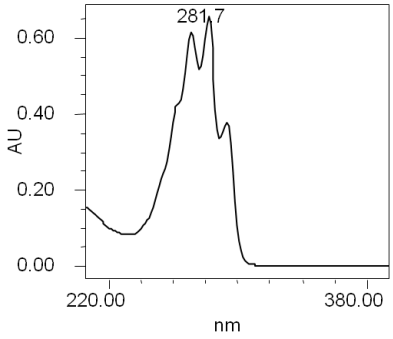

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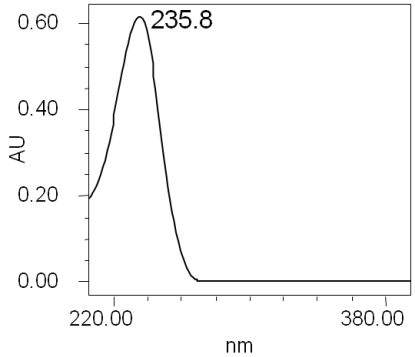
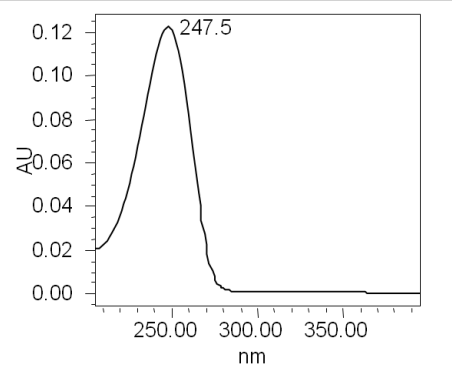


3β,5α-dihydroxycholest-7-en-6-one (DHCEO):



MS spectra (APCI) of isolated oxysterols from AY9944-treated rats:

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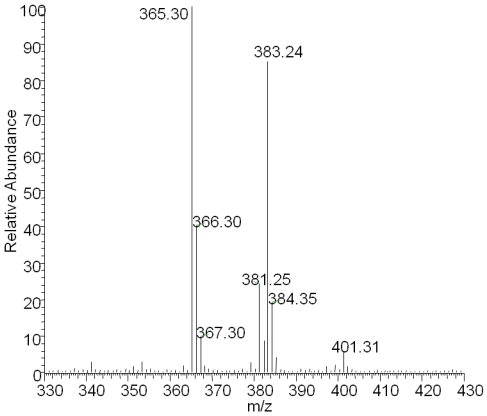

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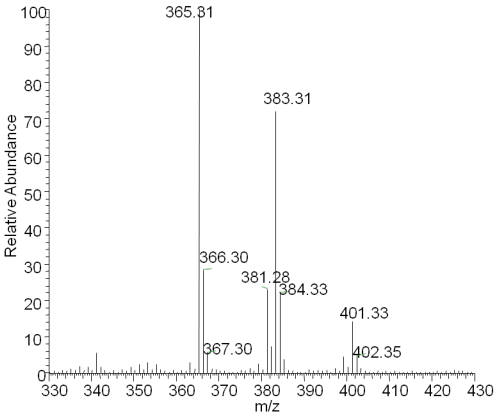

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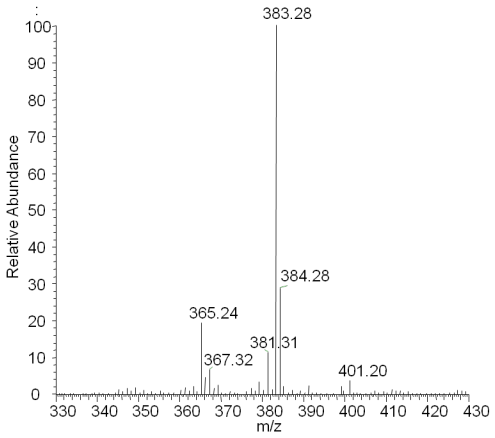

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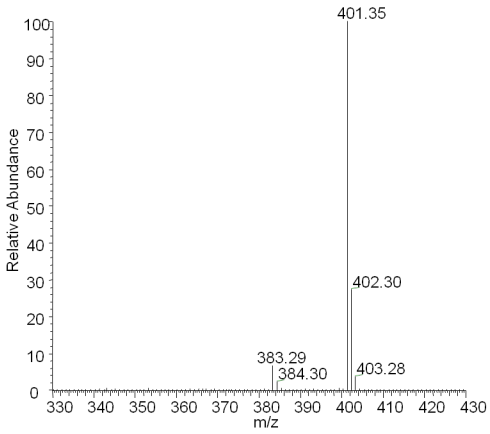

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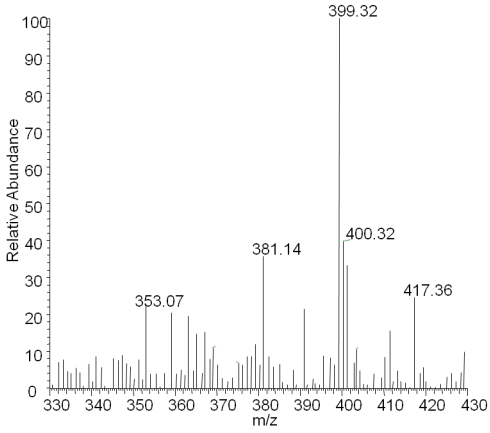

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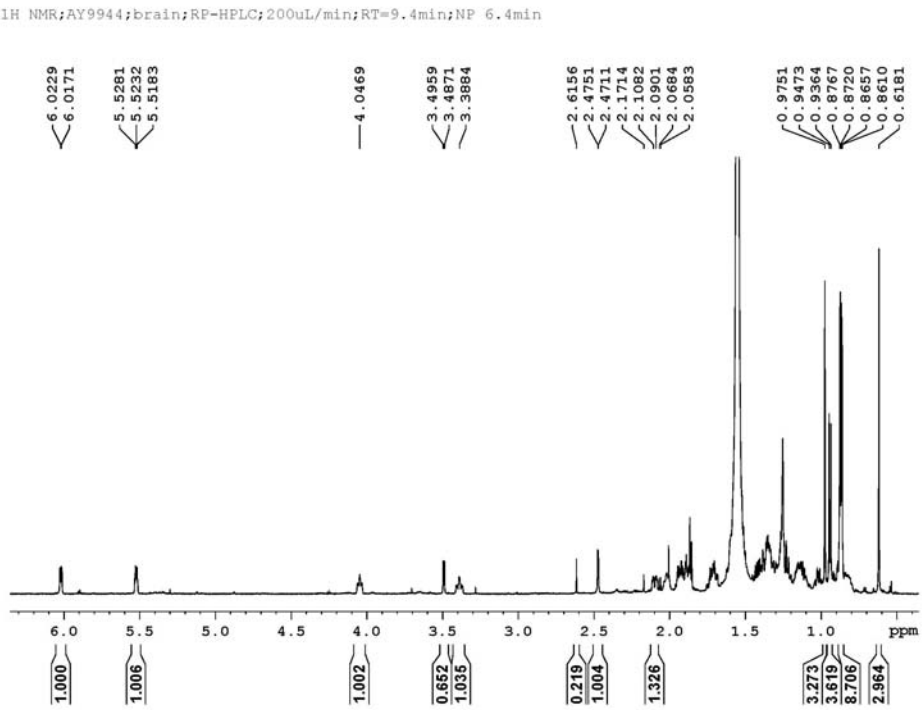
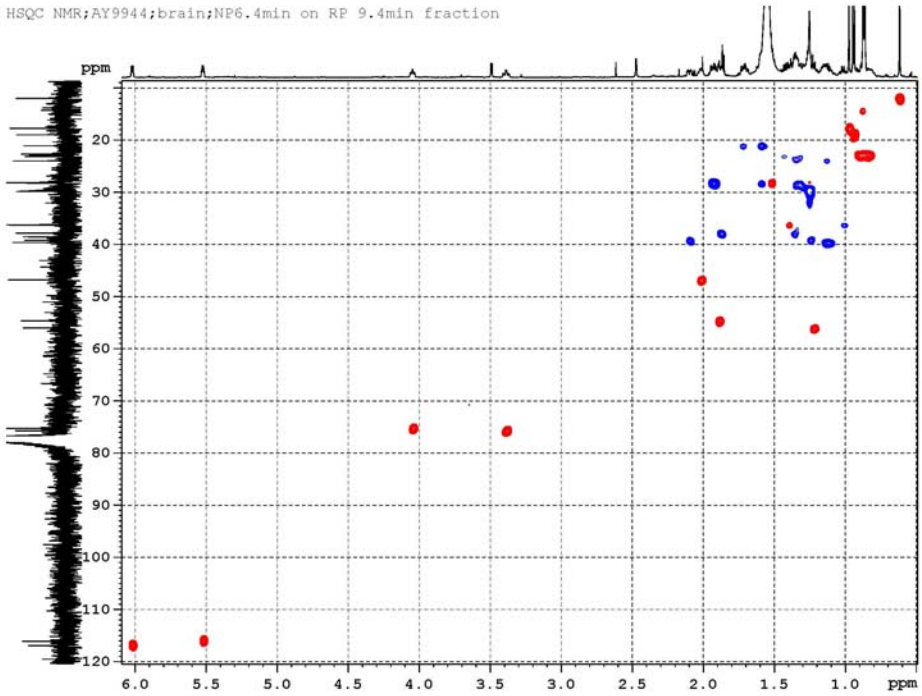
NMR Spectra of isolated oxysterols from AY9944-treated rats:

4α-hydroxy-7-DHC:

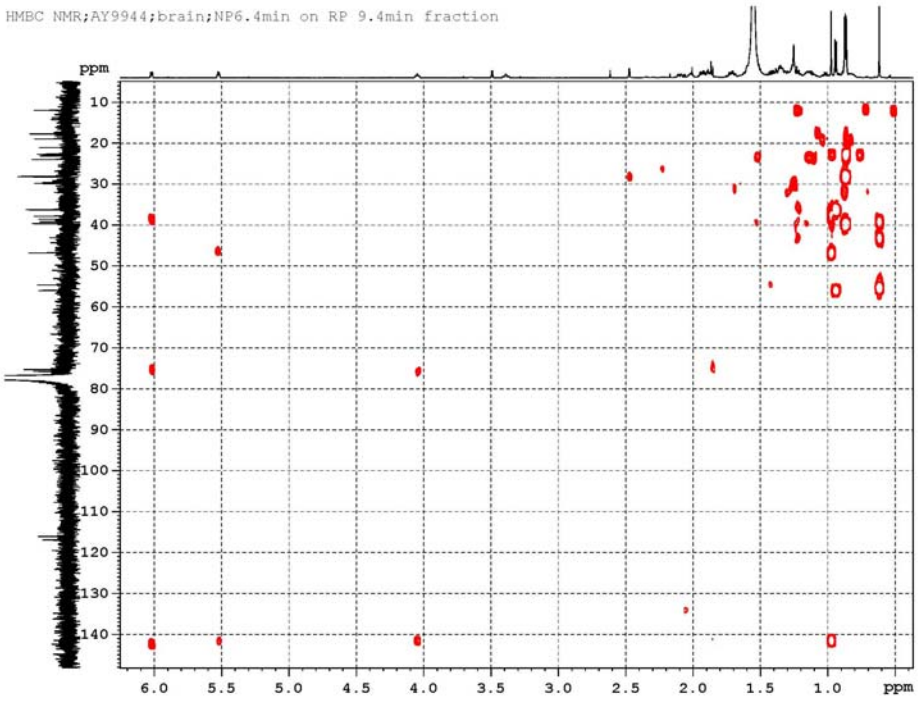
^{1}H NMR spectrum:



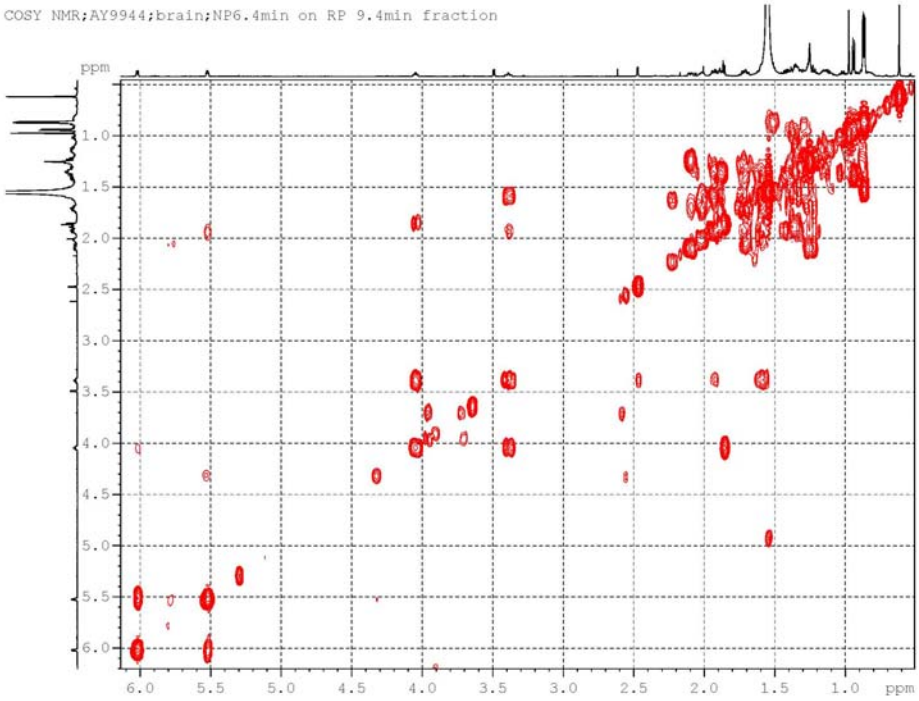
HSQC spectrum:



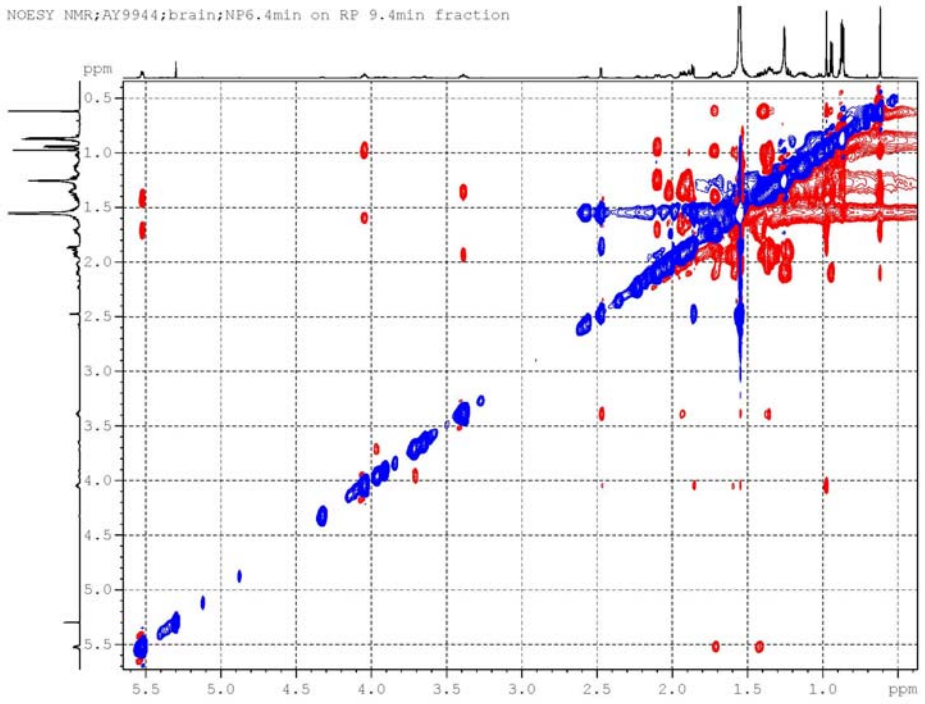
HMBC spectrum:



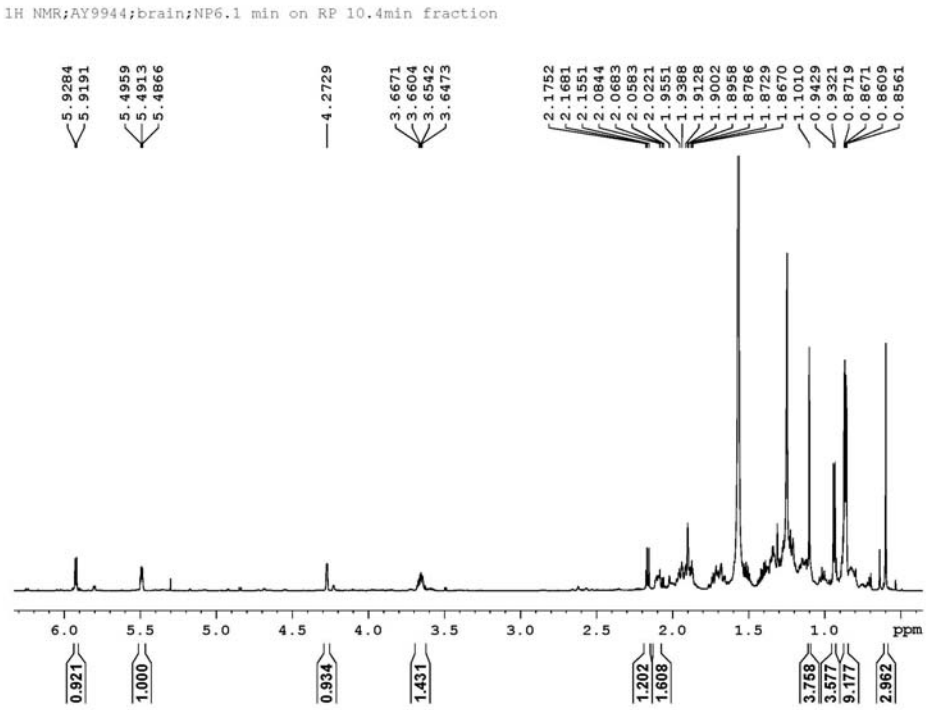
COSY spectrum:



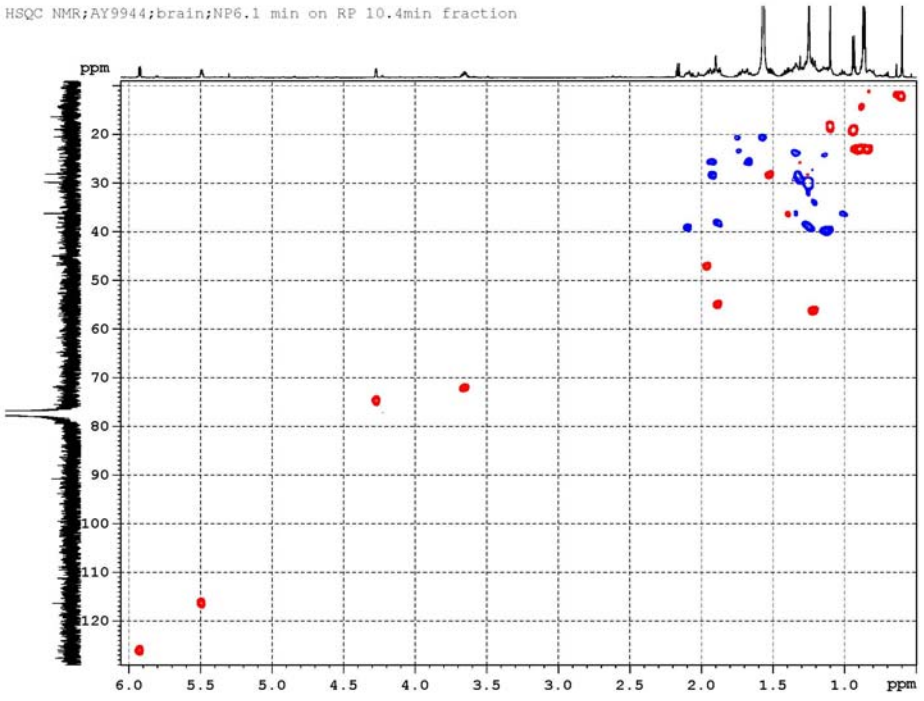
NOESY spectrum:



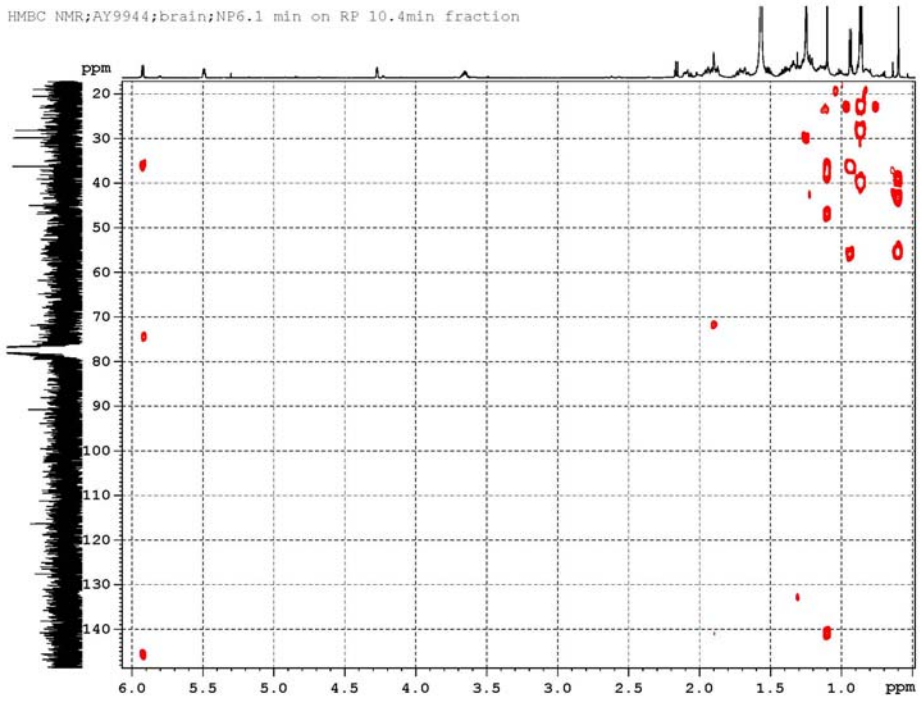
4β- hydroxy-7-DHC:
^{1}H NMR spectrum:



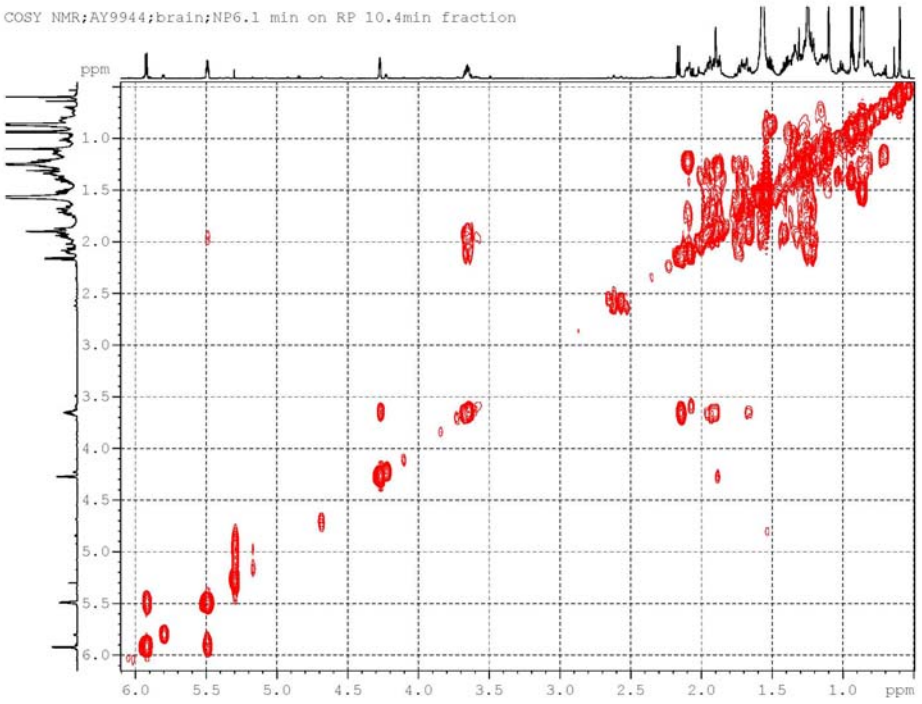
HSQC spectrum:



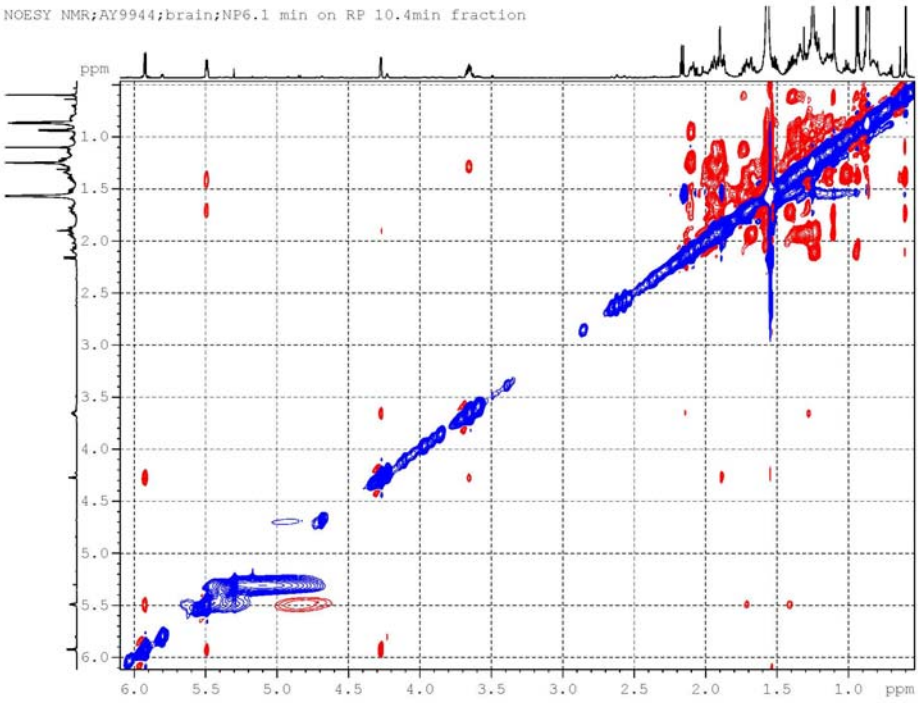
HMBC spectrum:



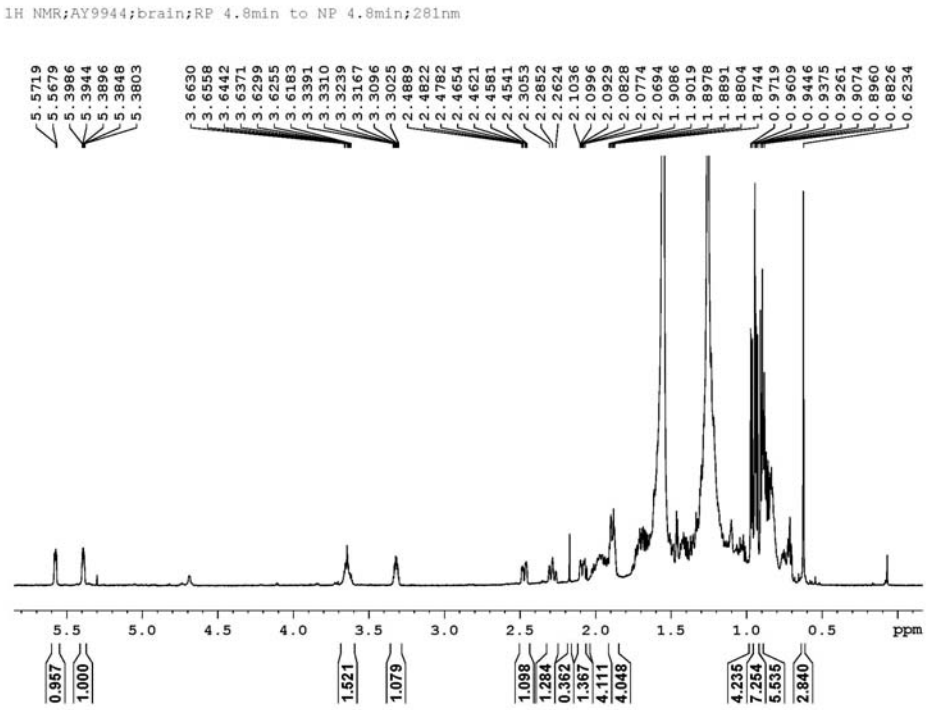
COSY spectrum:



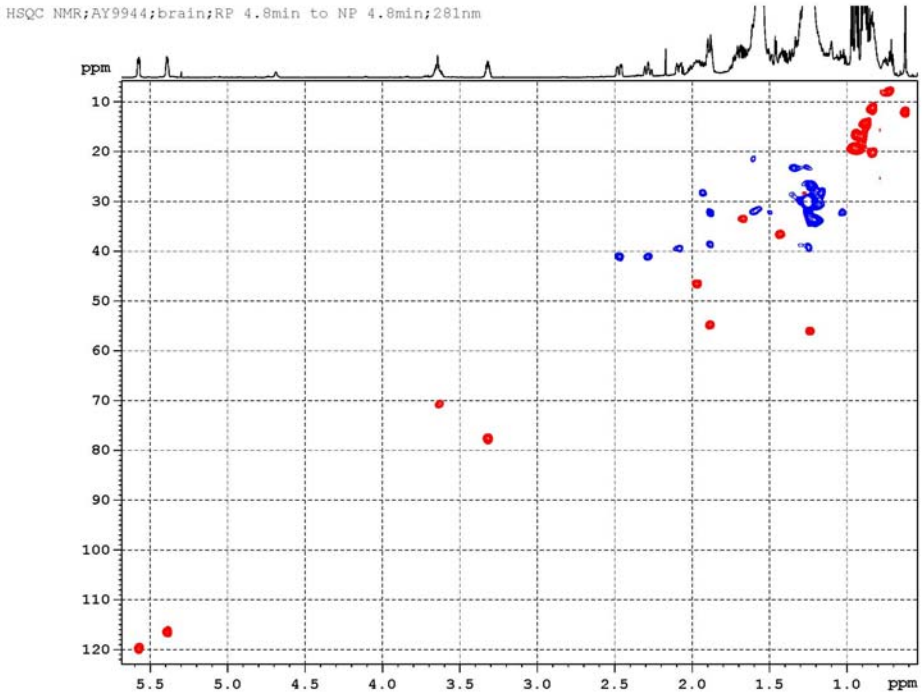
NOESY spectrum:



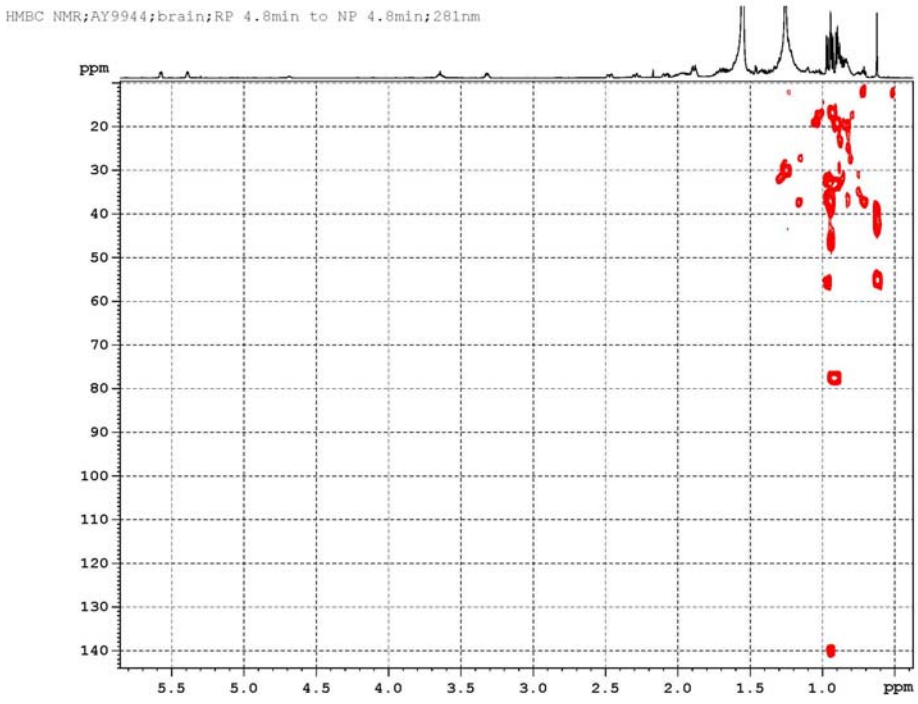
24-hydroxy-7-DHC:
^{1}H NMR:



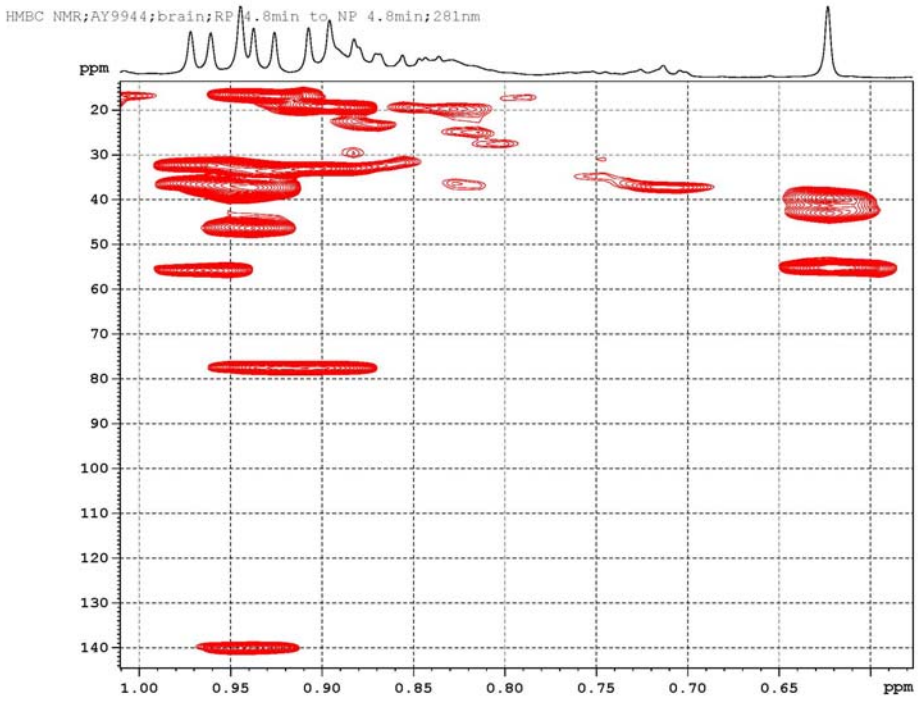
HSQC spectrum:



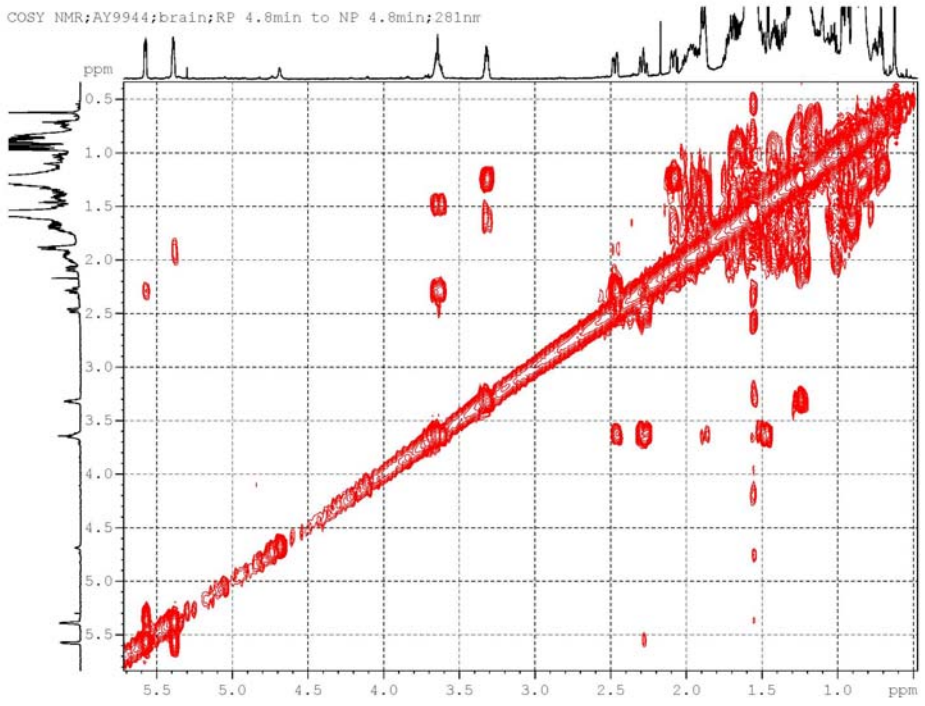
HMBC spectrum:



HMBC spectrum (expanded):

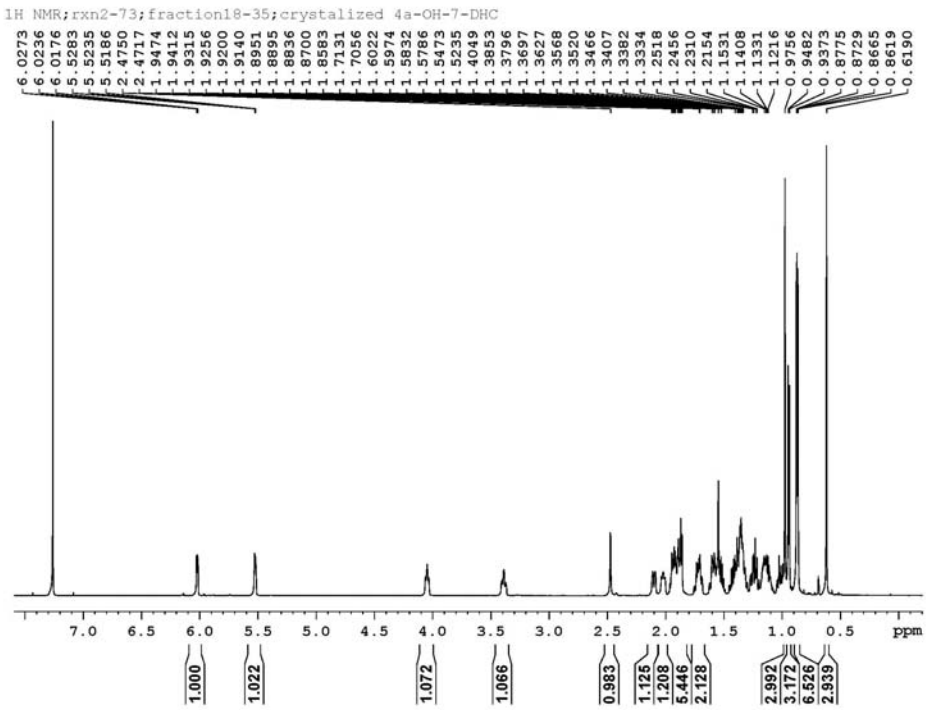


COSY spectrum:

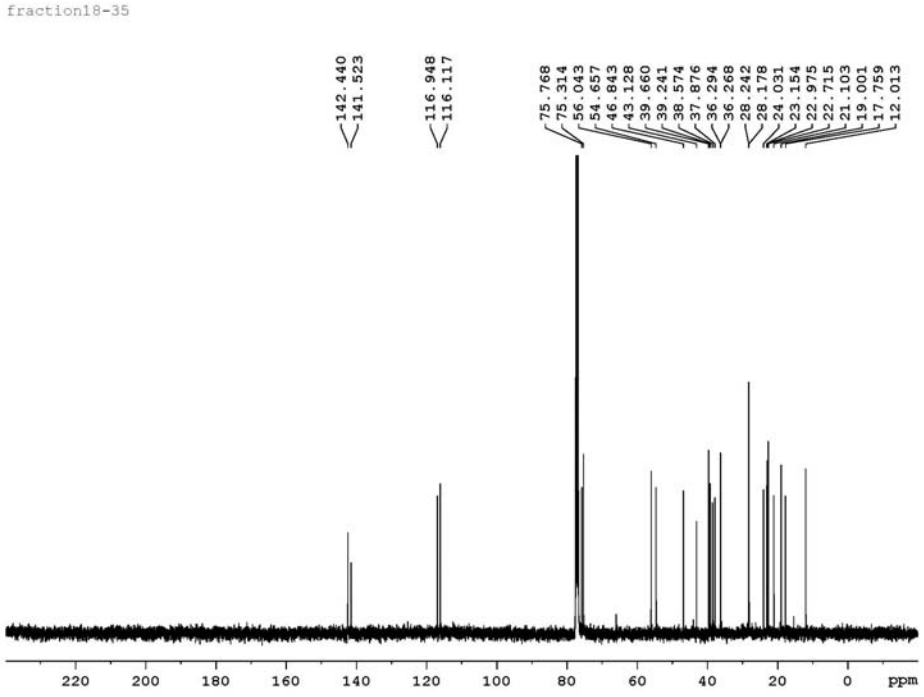


Synthetic 4α-hydroxy-7-DHC standard:

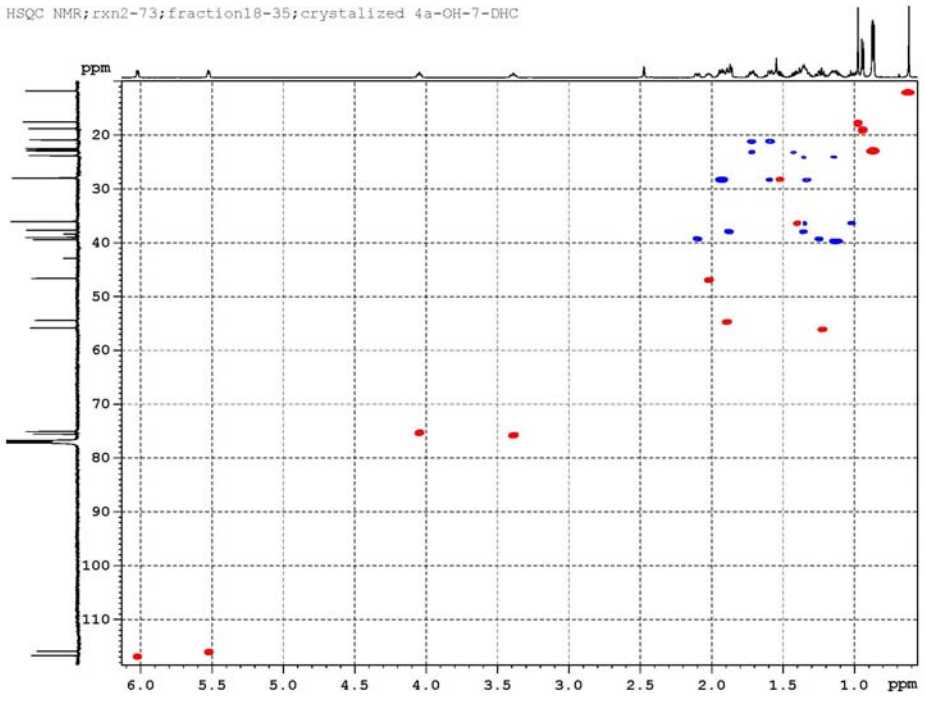
^{1}H NMR spectrum:



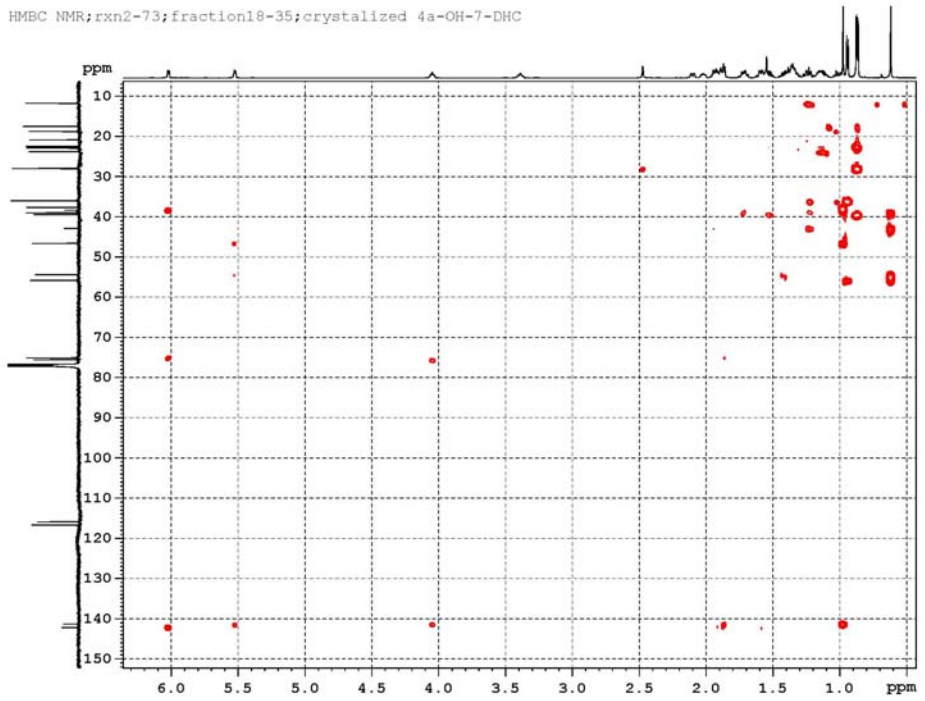
^{13}C NMR spectrum:



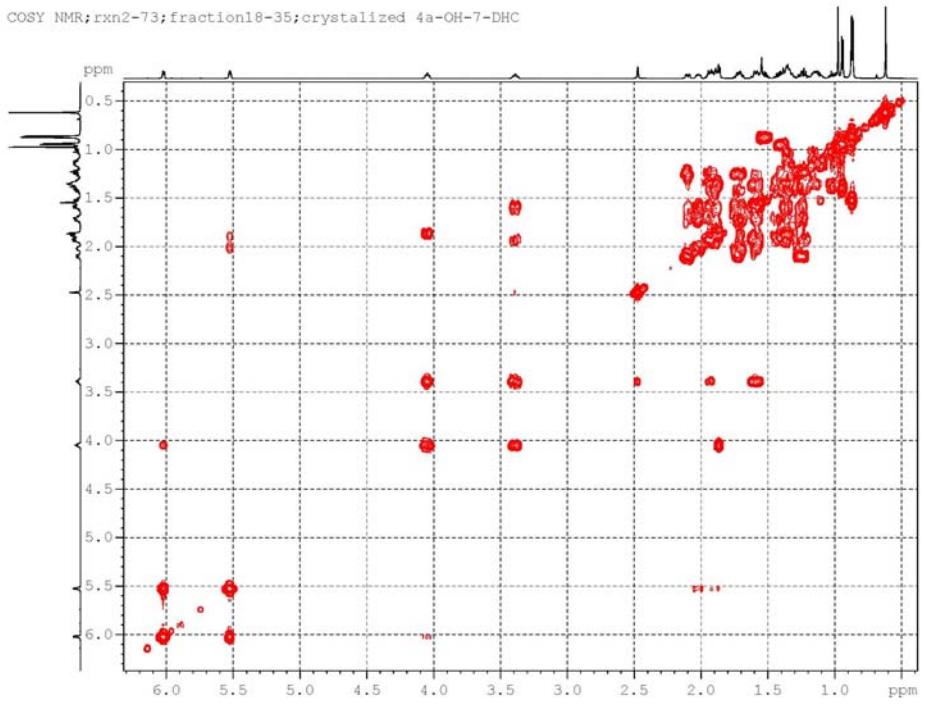
HSQC spectrum:



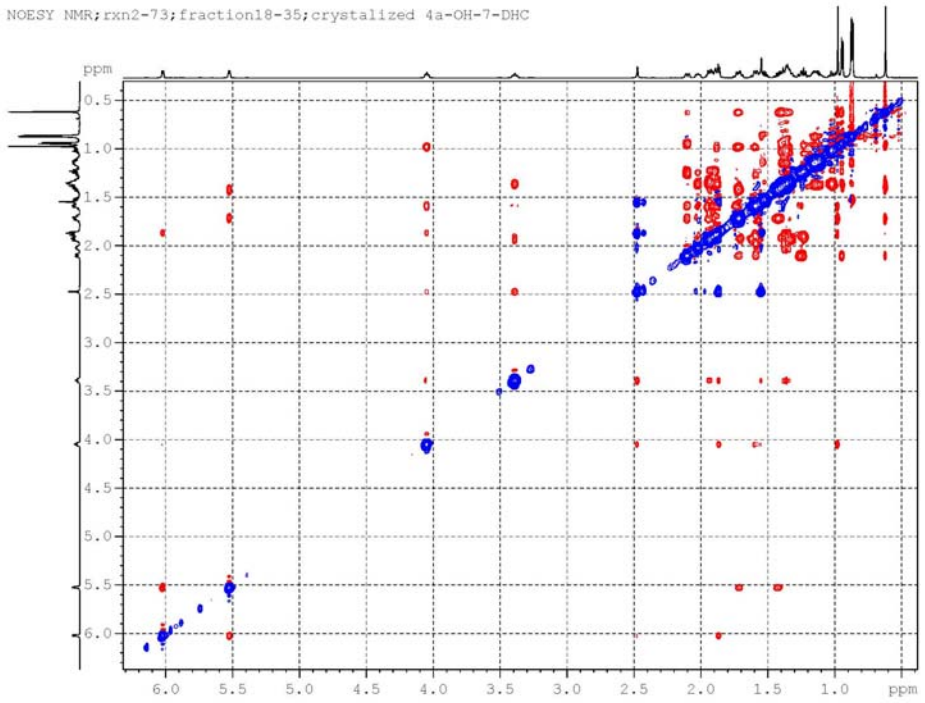
HMBC spectrum:



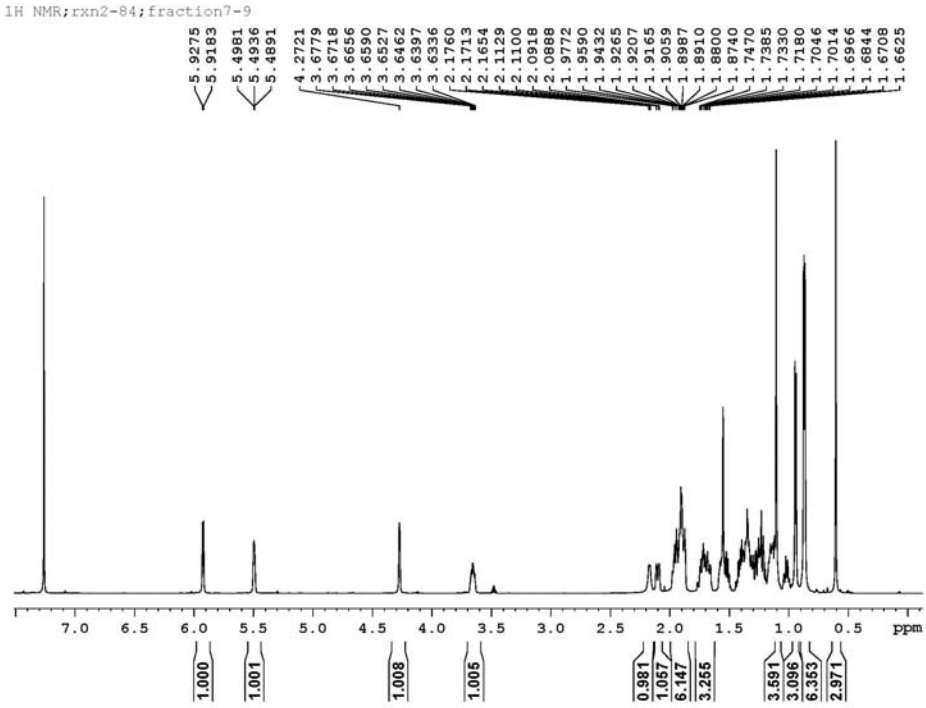
COSY spectrum:



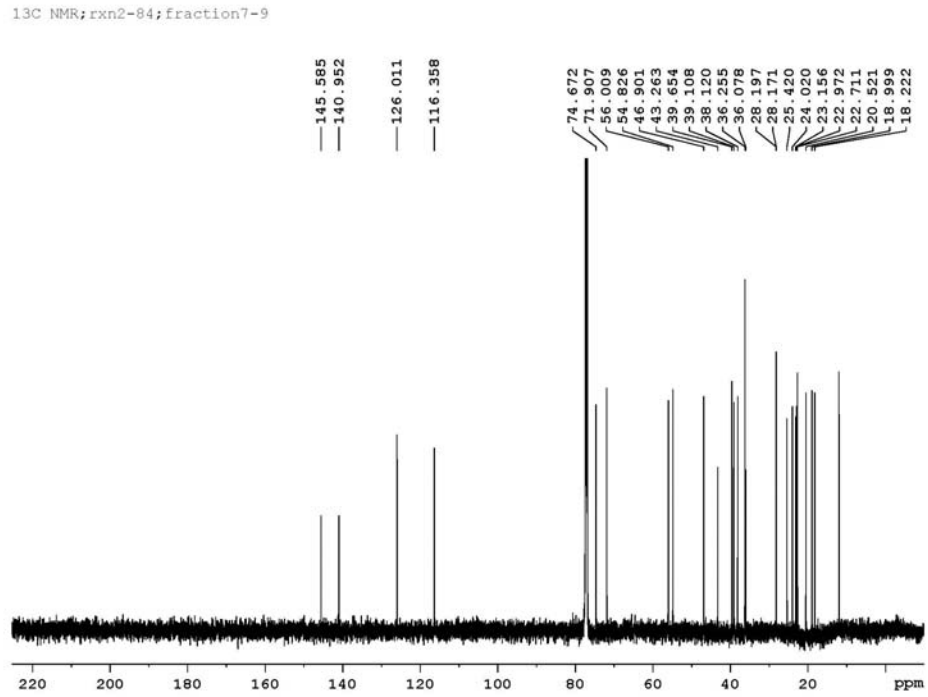
NOESY spectrum:



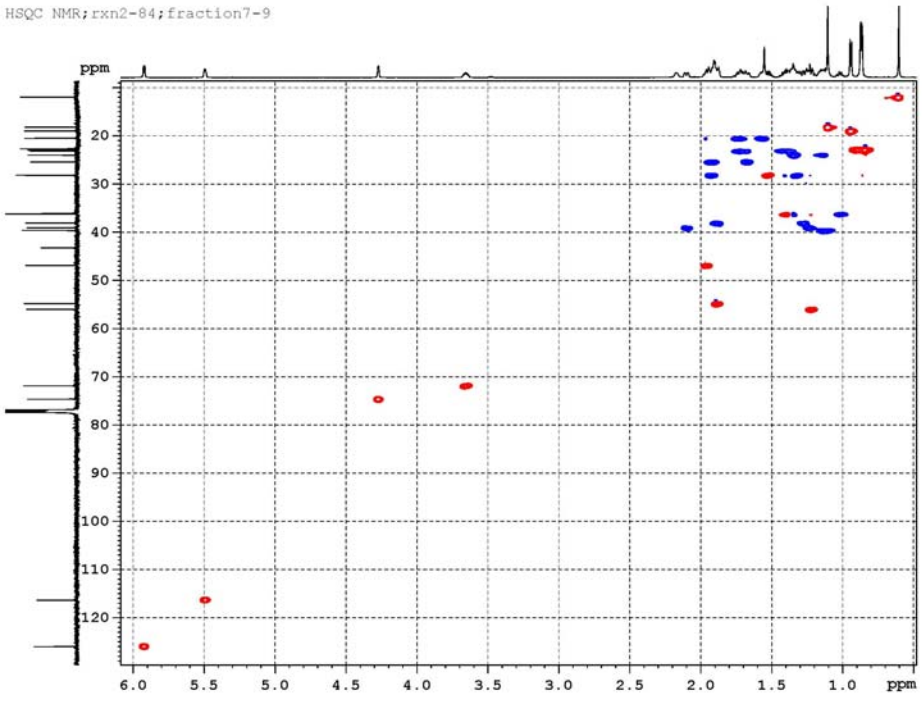
Synthetic 4β-hydroxy-7-DHC standard:
^{1}H NMR spectrum:



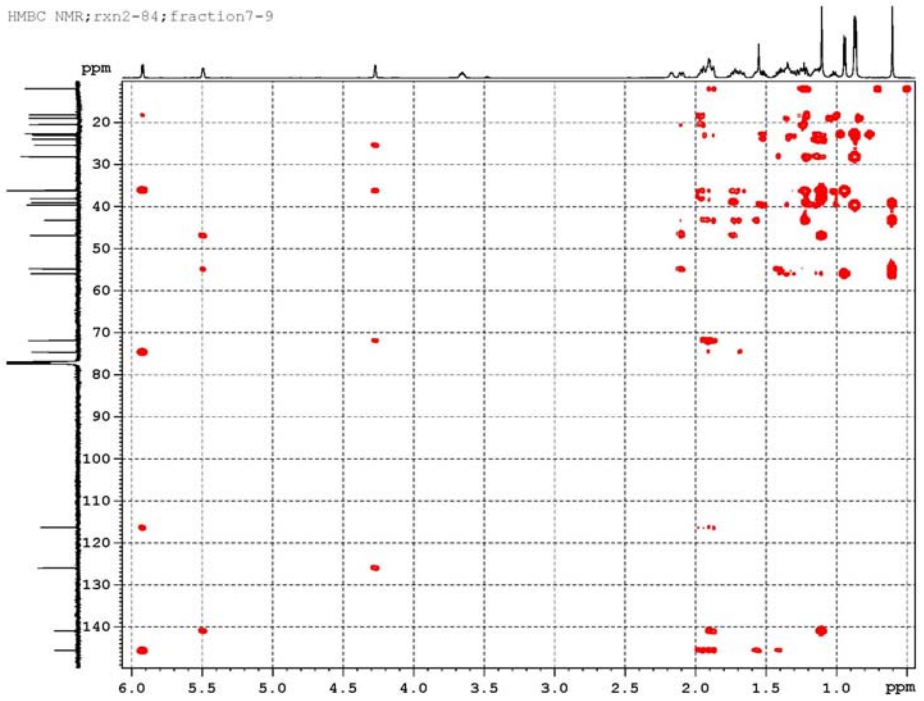
^{13}C NMR spectrum:



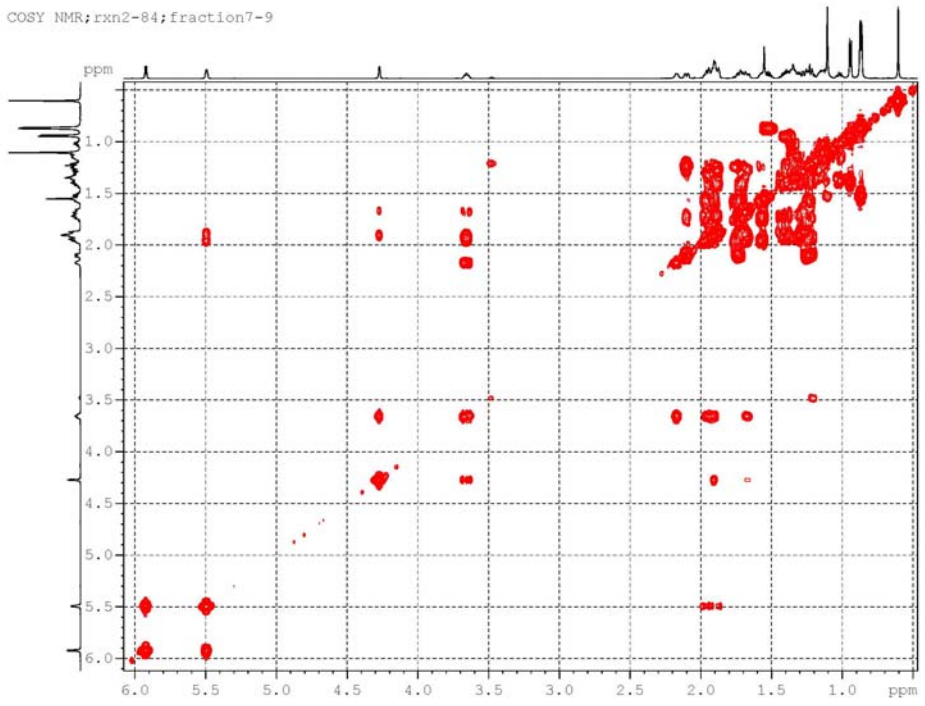
HSQC spectrum:



HMBC spectrum:



COSY spectrum:



NOESY spectrum: